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FORMULATION AND USE OF RETINOIDS IN TREATMENT OF

TITLE (FRENCH):

AND OTHER DISEASES
FORMULATION ET UTILISATION DE RETINOIDES DANS LE
TRAITEMENT DU CANCER ET D'AUTRES MALADIES
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ABEN . . . for therapeutic administration of retinoid to an animal. This method, in a preferred embodiment, comprises the basic steps of: preparing **liposomes** comprising phospholipid and **retinoid**; and parenterally administering a quantity of the resultant liposomes to the animal, said quantity containing a therapeutically effective amount of. . . . phosphatidic acid. These phospholipids, their derivatives and those of analogous structure and hydrophobic properties may be used to prepare the **liposome**-encapsulated **retinoids** of the present invention as would be apparent to one skilled in the relevant arts upon examination of the present descriptions. The phospholipids of these **retinoid**-containing **liposomes**, in a preferred embodiment, comprise dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, still more preferably in about a 7:3 ratio.

DETD Figure 8 shows dose-dependent effects of recombinant **interferon**-gamma (rIFN-g) on induction of tissue TGase activity in HPBM subpopulations. Small (O) and large (O) monocytes were cultured in serum. . . .

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Recent studies by the present inventors have shown that serum retinoids (vitamin A and analogs) exert a strong inhibitory effect on gamma-**interferon**-lipopolysaccharide (LPS) triggered cytostatic activation of murine macrophages: (Ks Mehtar et al., J. Immunol., June 1987, and incorporated herein by reference). Retinoid induced 5 suppression. . . of secretion of tumor necrosis factor (TNF-alpha)-. In these studies, mouse peritoneal macrophages were activated in the presence of either intact serum or de-lipidized serum supplemented with **retinoids**. The cells were metabolically labeled with S-methionine. Immunoprecipitation with anti-TNF antibodies followed by SDS polyacrylamide gel electrophoresis showed, in addition to a major. . .

human peripheral blood monocytes (HPBM) to macrophage-like cells was associated with the induction and accumulation of a specific intracellular TGasej tissue TGase [19,22]. Gamma (g)-**interferon**, which promotes the tumoricidal properties in HPBM, also augmented the expression of tissue TGase [191, Similalryr the activation of guinea pig and. . . subpopulations, show no significant difference in their ability to express tissue TGase activity induced by either in vitro culture or exposure to recombinant **interferon** gamma (rIFN-g), and that the expression of tissue TGase in cultured HPBM may be induced by a direct delivery of retinoids to. . .

RPMI-1640 medium supplemented with L-glutamine and human AB serum were from Gibco Laboratories (Grand Island, NY); Escherichia coli-derived human recombinant g- **interferon** (rIFN-g) was kindly supplied by Genentech Inc.

and liposome- encapsulated muramyl dipeptide by human peripheral blood monocytes. J. Reticuloendothelial Soc. 32,155, 1982
19* Mehta, K., Lopez-Berestein, G., Moore, W.T., and Davies, P.J.A. **Interferon**-g requires serum retinoids to promote the expression of tissue transglutaminase in cultured human blood monocytes. J. Immunol, 134r2053r 1985a
20e Mehta, K., Murtaugh, M.P., . . .



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<p>(21) International Application Number: PCT/US89/00435 (22) International Filing Date: 3 February 1989 (03.02.89)</p> <p>(31) Priority Application Number: 152,183 (32) Priority Date: 4 February 1988 (04.02.88) (33) Priority Country: US</p> <p>(71) Applicant: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).</p> <p>(72) Inventors: MEHTA, Kapil ; 8711 Ilona, Houston, TX 77025 (US). PEREZ-SOLER, Roman ; 3023 South Braeswood, Houston, TX 77025 (US). LOPEZ-BERESTEIN, Gabriel ; 5630 Rutherglen, Houston, TX 77096 (US).</p>		<p>(74) Agent: HODGINS, Daniel, S.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).</p> <p>(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).</p> <p>Published With international search report.</p>																									
<p>(54) Title: FORMULATION AND USE OF RETINOIDS IN TREATMENT OF CANCER AND OTHER DISEASES</p> <p>(57) Abstract</p> <p>The present invention involves a method for therapeutic administration of retinoid to an animal. This method, in a preferred embodiment, comprises the basic steps of: preparing liposomes comprising phospholipid and retinoid; and parenterally administering a quantity of the resultant liposomes to the animal, said quantity containing a therapeutically effective amount of the retinoid. The animal being administered the liposomes may bear a tumor impeded by retinoids and the administering step serve to impede growth of said tumor. The most preferred retinoid is all-trans retinoic acid although other retinoids may prove useful. In certain cases, the retinoid may be retinol, particularly all trans-retinol. The phospholipids of the present invention may be one or more of phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, sphingomyelin and phosphatidic acid. These phospholipids, their derivatives and those of analogous structure and hydrophobic properties may be used to prepare the liposome-encapsulated retinoids of the present invention as would be apparent to one skilled in the relevant arts upon examination of the present descriptions. The phospholipids of these retinoid-containing liposomes, in a preferred embodiment, comprise dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol, still more preferably in about a 7:3 ratio.</p>																											
<table border="1"> <caption>Data points estimated from the graph</caption> <thead> <tr> <th>Retinoic Acid Concentration (ng/ml)</th> <th>L-RA Growth Inhibition (%)</th> <th>RA Growth Inhibition (%)</th> <th>Empty Liposome Growth Inhibition (%)</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>0</td> <td>0</td> <td>0</td> </tr> <tr> <td>5</td> <td>~25</td> <td>0</td> <td>0</td> </tr> <tr> <td>10</td> <td>~75</td> <td>~40</td> <td>0</td> </tr> <tr> <td>15</td> <td>100</td> <td>~55</td> <td>~10</td> </tr> <tr> <td>20</td> <td>100</td> <td>~70</td> <td>~15</td> </tr> </tbody> </table>				Retinoic Acid Concentration (ng/ml)	L-RA Growth Inhibition (%)	RA Growth Inhibition (%)	Empty Liposome Growth Inhibition (%)	0	0	0	0	5	~25	0	0	10	~75	~40	0	15	100	~55	~10	20	100	~70	~15
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FORMULATION AND USE OF RETINOIDS
IN TREATMENT OF CANCER
AND OTHER DISEASES

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The present invention relates to therapeutic usage of retinoids encapsulated in liposomes.

20 It has been known for more than 50 years that retinoids, the family of molecules comprising both the natural and synthetic analogues of retinol (vitamin A), are potent agents for control of both cellular differentiation and cellular proliferation (Wolbach et al., J. 25 Exp. Med., 42:753-777, 1925). Several studies have shown that retinoids can suppress the process of carcinogenesis in vivo in experimental animals (for reviews, see e.g., Bollag, Cancer Chemother. Pharmacol., 3:207-215, 1979, and Sporn et al., In Zedeck et al. (eds.), Inhibition of Tumor 30 induction and development, pp. 71-100. New York: Plenum Publishing Corp., 1981). These results are now the basis of current attempts to use retinoids for cancer prevention in humans. Furthermore, there is an extensive evidence which suggests that retinoids can suppress the development 35 of malignant phenotype in vitro (for review, see e.g., Bertram et al., In: M.S. Arnott et al., (eds.), Molecular

- interactions of nutrition and cancer, pp 315-335. New York, Raven Press, 1982; Lotan et al., The modulation and mediation of cancer by vitamins, pp 211-223. Basel: S. Karger AG, 1983) thus suggesting a potential use of
5 retinoids in cancer prevention. Also, recently it has been shown that retinoids can exert effects on certain fully transformed, invasive, neoplastic cells leading in certain instances to a suppression of proliferation (Lotan, Biochim. Biophys. Acta, 605:33-91, 1980) and in
10 other instances to terminal differentiation of these cells, resulting in a more benign, non-neoplastic phenotype (see e.g., Brietman et al., Proc. Natl. Acad. Sci. U.S.A., 77:2936-2940, 1980).
- 15 Retinoids have also been shown to be effective in the treatment of cystic acne (see e.g., Peck, et al., New Engl. J. Med., 300:329-333, 1979). In addition to cystic acne, retinoid therapy has been shown to be effective in gram-negative folliculitis, acne fulminans, acne
20 conglobata, hidradenitis suppuritiva, dissecting cellulitis of the scalp, and acne rosacea (see e.g., Plewig et al., J. Am. Acad. Dermatol., 6:766-785, 1982).

However, due to highly toxic side effects of
25 naturally occurring forms of vitamin A (hypervitaminosis A) at therapeutic dose level, clinical use of retinoids has been limited (Kamm et al., In: The Retinoids. Sporn et al., (eds.), Academic Press, N.Y., pp 228-326, 1984; Lippman et al., Cancer Treatment Reports, 71:493-515,
30 1987). In free form, the retinoids may have access to the surrounding normal tissues which might be the basis of their profound toxicity to liver, central nervous system and skeletal tissue.

35 Therefore, one potential method to reduce the toxicity associated with retinoid administration would be

the use of a drug delivery system. The liposomal format is a useful one for controlling the topography of drug distribution in vivo. This, in essence, involves attaining a high concentration and/or long duration of 5 drug action at a target (e.g. a tumor) site where beneficial effects may occur, while maintaining a low concentration and/or reduced duration at other sites where adverse side effects may occur (Juliano, et al., In: Drug Delivery Systems, Juliano ed., Oxford Press, N.Y., pp 10 189-230, 1980). Liposome-encapsulation of drug may be expected to impact upon all the problems of controlled drug delivery since encapsulation radically alters the pharmacokinetics, distribution and metabolism of drugs.

15 The present invention involves a method for therapeutic administration of retinoid to an animal. This method, in a preferred embodiment, comprises the basic steps of: preparing liposomes comprising phospholipid and retinoid; and administering a quantity of the resultant 20 liposomes to the animal, said quantity containing a therapeutically effective amount of the retinoid. The retinoids may be administered parenterally, topically, orally or intraperitoneally. The animal being administered the liposomes may bear a tumor impeded by 25 retinoids and the administering step serve to impede growth of said tumor or the animal may have a dermatological disorder, ophthalmic disease, rheumatic disease or vitamin deficiency responsive to retinoids wherein the administering step results in clinical improvement. The 30 most preferred retinoid is all-trans retinoic acid although other retinoic acids may prove useful. In certain cases, the retinoid may be retinol, particularly all trans-retinol.

35 The phospholipids of the present invention may be one or more of phosphatidylcholine, phosphatidylserine,

phosphatidylglycerol, sphingomyelin and phosphatidic acid. These phospholipids, their derivatives, and those of analogous structure and hydrophobic properties may be used to prepare the liposome-encapsulated retinoids of the present invention as would be apparent to one skilled in the relevant arts upon examination of the present descriptions. In addition, the liposomes may also comprise a sterol component, for example, cholesterol. The phospholipids of these retinoid-containing liposomes, in a preferred embodiment, comprise dimyristoyl-phosphatidylcholine and dimyristoylphosphatidylglycerol, still more preferably in about a 7:3 ratio.

The processes of the present invention are particularly useful as a method for therapy or prophylaxis of an animal afflicted with cancer. Such a method may comprise: identifying an animal afflicted with cancer; preparing liposomes comprising phospholipid and a retinoid; and parenterally administering a quantity of said liposomes to the animal, said quantity containing a therapeutically effective amount of the retinoid.

From another view, the present invention may also comprise a method of inducing cellular differentiation. Such induced differentiation may be useful to impede proliferation of undifferentiated neoplastic cells or to promote the differentiation of normal cells having the potential differentiated capacity to attack neoplastic cells. More particularly as to the latter use, the liposome-encapsulated retinoids of the present invention may, for example, be used for inducing the in vivo differentiation of peripheral blood monocytes. This particular method comprises the steps of: preparing liposomes comprising phospholipid and retinoid; and parenterally administering a quantity of said liposomes to an animal, said quantity containing an amount of the

retinoid effectively inducing peripheral blood monocyte differentiation.

- The present invention further includes a process for
- 5 producing a powder which forms liposomes comprising a retinoid upon suspension of the powder in an aqueous solution. This process comprises the steps of: dissolving retinoid in t-butanol to form a first solution, mixing the first solution and a dry phospholipid to form a
- 10 second solution, and lyophilizing the second solution to produce a powder. In a preferred embodiment, the phospholipids are defined as a phospholipid film. The solution or powder preferably has a ratio of retinoid to phospholipid between about 1:5 and about 1:50, more
- 15 preferably between about 1:10 and about 1:15. A composition of matter produced essentially by this process is also an object of this invention. A reconstituted liposomal retinoid preparation may be produced by simple agitation of the above powder in an aqueous solution.
- 20 Such a reconstituted liposomal retinoid preparation may be used for therapy or prevention by parenteral administration.

- The term "liposomes" as used herein means man-made
- 25 lipid vesicles which may include plurilamellar lipid vesicles, stable plurilamellar vesicles, small sonicated multilamellar vesicles, reverse phase evaporation vesicles, large multilamellar lipid vesicles, and so forth.

30

Figure 1 shows a time profile of liposomal retinoic acid (L-RA) stability in the presence (●) and absence (○) of serum.

35

Figure 2 shows human red blood cell (RBC) lysis as a function of time with RA (●) and L-RA (▲).

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Figure 3 shows RBC lysis as a function of retinoic acid (RA) concentration (●) and L-RA concentration (▲).

Figure 4 shows the inhibition of THP-1 cell growth as
5 a function of RA concentration (●), L-RA concentration
(○) or empty liposome concentration (Δ).

Figure 5 shows the induction of transglutaminase
(TGase) in human monocytic THP-1 cells as a function of
10 treatment with RA (▨) or L-RA (■).

Figure 6 shows the inhibition of human histiocytic
U-937 cell growth as a function of RA concentration (●),
L-RA concentration (○) and empty liposome concentration
15 (Δ).

Figure 7 shows the time course of accumulation of
tissue TGase activity in cultured human peripheral blood
monocytes (HPBM). HPBM were fractionated into small (○)
20 and large (●) subpopulations by centrifugal elutriation,
and they were cultured in 35-mm-well tissue culture plates
as described in Materials and Methods. At the indicated
time points the cells were washed, sonicated, and assayed
for TGase activity. Values are the means of six deter-
25 minations from two dishes.

Figure 8 shows dose-dependent effects of recombinant
interferon-gamma (rIFN- γ) on induction of tissue TGase
activity in HPBM subpopulations. Small (○) and large
30 (●) monocytes were cultured in serum containing medium
alone or medium containing increasing concentrations of
rIFN- γ . After 72 hr, the cells were harvested and the
cell lysates assayed for tissue TGase activity. The
results shown represent mean \pm SD of three determinations
35 from an individual donor.

Figure 9 shows effects of retinol (ROH) and RA on induction of tissue TGase activity in cultured HPBM. Cells were cultured in the presence of 5% human AB serum and the absence (O) or presence of 500 nM ROH (Δ) or RA 5 (\bullet) for varying periods of time. At the end of each time point, the cells were harvested and assayed for enzyme activity. Values shown are the means \pm SD of six determinations from two independent experiments. Inset, dose-response curve for tissue TGase induction by ROH (\blacktriangle) and 10 RA (\bullet) in HPBM after 72-hr culture.

Figure 10 shows effects of free- and liposome-encapsulated RA on induction of tissue TGase in HPBM.
A: The cells were cultured in tissue culture dishes in 15 presence of serum-containing medium alone (Δ) 500 nm liposomal RA (\bullet), or medium containing 500 nM free-RA (Δ), or "empty liposomes" (O) for indicated periods of time. Both the liposomal RA and "empty liposomes" contained 200 ug/ml lipid. At the end of each time point, 20 the cultures were washed and cell lysates assayed for TGase activity. Values shown are the mean \pm SD of six determinations from two independent experiments.
B: Western-blot analysis of the levels of tissue TGase in freshly isolated HPBM (lane 1) and in HPBM cultured for 72 25 hr in the presence of serum-containing medium alone (lane 2), in medium containing 500 nM free RA (lane 3), 500 nM liposomal RA (lane 4), or "empty liposomes" (lane 5). Cell lysates containing 25 ug of protein were subjected to Western-blot analysis as described in Materials and 30 Methods.

Figure 11 shows effect of free and liposome-encapsulated ROH on induction of tissue TGase in HPBM.
A: HPBM monolayers were cultured in serum-containing 35 medium alone (Δ) or medium containing 1 uM of free- (O) or liposomal-ROH (\blacktriangle) for 72 hr. Then the cultures were

washed and the cell lysates assayed for enzyme activity as described in Materials and Methods. B: Western-blot analysis of tissue TGase levels in freshly isolated HPBM (lane 1) and in HPBM cultured for 72 hr in the presence of 5 serum-containing medium alone (lane 2), in medium containing 1 uM of free ROH (lane 3), or liposome-encapsulated ROH (lane 4) as described in Materials and Methods. Twenty-five micrograms of cell protein was loaded onto each lane.

10

In a broad and general sense, the present invention relates to liposome-encapsulated retinoids. As generally known, the lipid membranes of liposomes are formed of a bimolecular layer of one or more naturally occurring 15 and/or synthetic lipid compounds having polar heads and nonpolar tails. The present inventors have shown that the encapsulation of retinoic acid in liposomes decreases the toxicity observed with use of the free drug.

20

Representative, suitable compounds for forming liposomes useful in the present invention are phosphatidylcholine, both naturally occurring and synthetically prepared, phosphatidic acid, phosphatidylserine, phosphatidylglycerol, sphingolipids, 25 sphingomyelin, cardiolipin, glycolipids, gangliosides, cerebrosides, phosphatides, sterols, and the like.

More particularly useful phospholipids include dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol. In addition, the following compounds may 30 be suitable: egg phosphatidylcholine, dilaurylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, 1-myristoyl-2-palmitoylphosphatidylcholine, 1-palmitoyl-2-myristoylphosphatidylcholine, 1-palmitoyl-2-stearoylphosphatidylcholine, 1-stearoyl-2-palmitoyl

- phosphatidylcholine, dioleoylphosphatidylcholine,
dilauryloylphosphatidylglycerol,
dipalmitoylphosphatidylglycerol,
distearoylphosphatidylglycerol,
5 dioleoylphosphatidylglycerol, dimyristoylphosphatidic
acid, dipalmitoyl phosphatidic acid, dimyristoyl
phosphatidylethanolamine, dipalmitoyl
phosphatidylethanolamine, dimyristoyl phosphatidylserine,
dipalmitoyl phosphatidylserine, brain phosphatidylserine,
10 brain sphingomyelin, dipalmitoyl sphingomyelin, and
distearoyl sphingomyelin.

In addition, other lipids such as steroids and cholesterol may be intermixed with the phospholipid components to confer certain desired and known properties on the resultant liposomes. Further, synthetic phospholipids containing either altered aliphatic portions, such as hydroxyl groups, branched carbon chains, cycloderivatives, aromatic derivatives, ethers, amides, 20 polyunsaturated derivatives, halogenated derivatives, or altered hydrophilic portions containing carbohydrate, glycol, phosphate, phosphonate, quaternary amine, sulfate, sulfonate, carboxy, amine, sulphydryl, imidazole groups and combinations of such groups, can be either substituted 25 or intermixed with the phospholipids.

Suitable therapeutic agents for encapsulation may include various retinoids. Although retinoic acid, more particularly trans-retinoic acid and retinol, more 30 particularly, all-trans-retinol, are preferred, it also believed that the following compounds, may be successfully encapsulated: all-trans-retinoic acid, retinoic acid methyl ester, retinoic acid ethyl ester, phenyl analog of retinoic acid, etretinate, retinol, retinyl acetate, 35 retinaldehyde, and 13-cis-retinoic acid. These compounds and their pharmacologic activities are described in

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Roberts and Goodman, Biological Methods for Analysis and Assay of Retinoids: Relationship Between Structure and Activity In: Retinoids, Sporn and Roberts, Eds., Academy Press, p. 235, 1984.

5

The discoveries presented herein involve manifold uses of retinoids encapsulated in liposomes. For example, the encapsulation of retinoic acid in liposomes results into at least 15-fold decrease in the toxicity as compared
10 to the free drug.

The encapsulation of certain retinoids, such as retinol, within liposomes permits their direct delivery to the intracellular sites and thus circumvents the requirement for cell surface receptors. This may be of particular significance, for example, in therapy of tumors which lack the cell surface receptors for serum retinol binding protein but possess intracellular receptors for retinoic acid.
15

20

Encapsulation of retinoids within liposomes allows an intravenous administration of the drug. There has been no acceptable vehicle available to permit intravenous administration of retinoids. Due to the highly lipophilic character of retinoids the use of liposomes to deliver retinoids is an attractive approach to reduce their toxicity. Using multilamellar liposomes, retinoids can be efficiently encapsulated without losing their activity while reducing their toxicity by at least 15-fold.
25

30

The use of vitamin A and its analogues (Retinoids) in the prevention and treatment of human cancer represents a relatively new direction in oncologic therapeutics. Recent laboratory investigations have documented that the
35 retinoids can block phenotypic expression of cancer, whether initiated by chemical, viral, physical, or

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biologic carcinogens. In humans also, the retinoids have been shown to cause regression of premalignant lesions and leukoplakia. However, the use of retinoids has been associated with both short term toxicity such as central nervous system alterations, as well as chronic intoxication such as skin and mucous membrane dryness and liver impairment, which eventually become irreversible. A considerable amount of effort has been devoted to develop vitamin A derivatives with an improved therapeutic/toxicity ratio. The effort has met with very little success.

Human peripheral blood monocytes (HPBM) from normal donors, isolated by countercurrent centrifugal elutriation into two subpopulations, showed no significant difference in their ability in vitro to differentiate into macrophages, as determined by induction of the protein cross-linking enzyme, tissue transglutaminase (TGase). The two subpopulations were equally responsive to the augmenting effect of recombinant interferon-gamma (rIFN- γ) on expression of tissue TGase. In vitro maturation and treatment with rIFN- γ of HPBM were associated with increased binding of tritiated retinol. Intracellular delivery of retinol rendered this hormone active in inducing the differentiation of HPBM. The retinoid-induced expression of tissue TGase was the result of increased accumulation of the enzyme peptide and not activation of preexisting enzyme. Maturation of HPBM, induced by in vitro culture or treatment with rIFN- γ , appeared to be associated with acquisition of cell surface receptors for serum retinol-binding protein.

In addition, it is believed that the liposomal retinoids may be used as anti-inflammatory reagents. Recent studies by the present inventors have shown that serum retinoids (vitamin A and analogs) exert a strong

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inhibitory effect on gamma-interferon-lipopolysaccharide (LPS) triggered cytostatic activation of murine macrophages (K. Mehta, et al., J. Immunol., June 1987, and incorporated herein by reference). Retinoid induced suppression of macrophage activation is associated with induction and accumulation of a protein cross-linking enzyme, tissue transglutaminase. Recent studies suggest that the inhibition of cystostatic activation is mediated through inhibition of secretion of tumor necrosis factor (TNF-alpha). In these studies, mouse peritoneal macrophages were activated in the presence of either intact serum or de-lipidized serum supplemented with retinoids. The cells were metabolically labeled with ³⁵S-methionine. Immunoprecipitation with anti-TNF antibodies followed by SDS polyacrylamide gel electrophoresis showed, in addition to a major band at 17.5 Kd for TNF, the presence of high molecular weight bands (30Kd and 65 Kd). Moreover, an enzymatic assay, pure recombinant TNF-alpha in the presence of 5 mM Ca⁺² and purified tissue TGase from guinea pig liver incorporated large amounts of ³H-putrescine.

Taken together, these studies suggest that TNF-alpha can serve as an endogenous substrate for tissue TGase. Therefore, retinoid-induced expression of tissue TGase may cause inter- or intra-molecular cross-linking of TNF, thereby inactivating it or inhibiting its secretion into the extracellular environment. Since factors such as tumor necrosis factor and interleukin-1 (IL-1) (both are released by activated macrophages) are the main mediators of inflammation (Nawroth, et al., J. Exp. Med.; 163:1363-1375, 1986), by inhibiting the release of such mediators from macrophages, it may be possible to inhibit the whole cascade leading to inflammation.

- Indeed, retinoids have been shown to be effective as anti-inflammatory agents (Hensby, Agents and Actions, 21:238, 1987). Also, TNF has been shown to induce the release of IL-1 by endothelial cells (Dinarello, *et al.*, 5 J. Exp. Med., 163:1433-1439, 1986). In addition, certain retinoids (Etretinate and Isotretinoin) have been reported to inhibit neutrophil and monocyte migration in patients with dermatological disorders. Retinoids have also been shown to inhibit other discrete polymorphonuclear 10 leukocyte functions in vitro. It has been suggested that retinoids may exert their anti-inflammatory effects by interacting with neutrophil membranes to inhibit a variety of responses, such as lysosomal enzyme release and superoxide generation.
- 15
- In addition, retinoids have proven effective in treating a wide variety of dermatological diseases, including all types of acne, and recent studies have shown that topical administration of retinoids may be effective 20 in reversing UV-induced aging of the skin. Retinoids have also been used to treat rheumatic diseases, as immunomodulators (against cancer, infectious diseases, and parasitic diseases), as eye drops or ointments for preventing certain eye diseases, for treatment of vitamin 25 A deficiency disorder, and as dietary supplements. It is contemplated that the liposome encapsulated retinoids of the present invention will prove as effective in treating these diseases as the free retinoids, but will not have the associated toxicity. Therefore, use of liposomal 30 encapsulated retinoids should prove advantageous for treating these disorders and is considered to be within the scope of the present invention.

These examples are presented to describe preferred 35 embodiments and utilities of the present invention and are

not meant to limit the present invention unless otherwise stated in the claims appended hereto.

5

EXAMPLE 1Preparation of liposomal-all trans-retinoic acid (L-RA)

10 Preparation of lyophilized powder containing all trans-retinoic acid and phospholipids was carried out as follows.

A solution of retinoic acid in t-butanol (1-5 mg/ml) 15 was added to a dry lipid film containing dimyristoylphosphatidyl choline (DMPC) and dimyristoylphosphatidyl glycerol (DMPG) at a 7:3 molar ratio. The phospholipids were solubilized in the t-butanol containing the all-trans retinoic acid and the solution was freeze-dried overnight.

20 A powder containing dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), and all-trans retinoic acid was obtained. The lipid:drug ratio used was from 10:1 to 15:1.

25 Reconstitution of liposomal retinoic acid from the lyophilized powder was done as follows. The lyophilized powder was mixed with normal saline at room temperature to form multilamellar liposomes containing all trans-retinoic acid. This reconstitution method required mild hand- 30 shaking for 1 min to obtain a preparation devoid of any aggregates or clumps. By light microscopy, the reconstituted preparation contained multilamellar liposomes of a close size range. No aggregates or drug clumps were identified in the liposomal preparation in 35 three different experiments.

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Encapsulation efficiency and size distribution of the liposomal all-trans retinoic acid preparation were determined as follows.

5 The liposomal all-trans retinoic acid preparation was centrifuged at 30,000 x g for 45 minutes. A yellowish pellet containing the retinoic acid and the lipids was obtained. By light microscopy, the pellet was composed of liposomes with no crystals or drug aggregates. The
10 encapsulation efficiency was calculated to be greater than 90% by measuring the amount of free retinoic acid in the supernatant by UV spectrophotometry. Liposomes were sized in a Coulter-Counter and Channelizer. The size distribution was as follows: 27% of liposomes less than 2
15 micrometers (μm), 65% between 2 μm and 3 μm , 14% between 3 μm and 5 μm , 1% more than 5 μm . The method used for encapsulation of retinoids was simple, reproducible and could be used for large scale production, for example, for clinical trials.

20 Further experiments were performed by the same procedure but with different lipids, ratios of lipids and the use of ^3H -all-trans retinoic acid. Additional lipids utilized were dipalmitoylphosphatidylcholine (DPPC)
25 stearylamine (SA) and cholesterol. After sedimentation of the liposomes, residual ^3H was determined and encapsulation efficiency calculated. Table 1 shows encapsulation efficiencies determined by this method for various L-RA preparations.

30

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TABLE 1

5
Encapsulation Efficiency of
Retinoic Acid in Liposomes

	LIPOSOME COMPOSITION	ENCAPSULATION EFFICIENCY (%)
10	DMPC:Cholesterol 9:1	69.3
	DMPC:Cholesterol 9:3	64.5
15	DPPC	69.1
	DMPC;SA:Cholesterol 8:1:1	56.7
20	DMPC:DMPG 7:3	90
	DMPC:DMPG 9:1	90.7
25		

30 Of the lipid compositions studies, DMPC:DMPG at ratios between 7:3 and 9:1 gave superior encapsulation efficiencies.

35 Liposomal all-trans retinol (L-ROH) was prepared by the methods described above for L-RA with DMPC:DMPG, 7:3.

35 EXAMPLE 2

Stability of Liposomal Retinoic Acid

40 Liposomal ^3H -retinoic acid (L- ^3H -RA) was prepared with DMPC:DMPG, 7:3 as described in Example 1. Samples of the L- ^3H -RA were incubated with either phosphate-buffered saline (PBS) or PBS with 20% (by volume) fetal calf serum (FCS). After various periods of incubation at about 37°C,

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aliquots were removed and centrifuged to sediment liposomes. The tritium in the supernatant solution measured to determine ^3H -RA release. Figure 1 shows the release of ^3H -RA over a two day period. The L- ^3H -RA was 5 over about 80% stable over the period of the experiment, even in the presence of 20% FCS.

When ^3H -all-trans retinol was used to label L-ROH and stability in PBS measured, only about 5% of the ^3H -ROH was 10 released after a 24 hr incubation at 37°C.

EXAMPLE 3

15 In Vitro Lysis of Human Erythrocytes (RBCs)
 by Retinoic Acid or Liposomal Retinoic Acid

Lysis of human red blood cells (RBCs) was quantitated by measuring the release of hemoglobin in the supernatants 20 by observation of increases in optical density at 550 nanometers (nm), as described previously (Mehta, et al., Biochem. Biophys. Acta., Vol. 770-, pp 230-234 (1984). Free-RA dissolved in dimethyl formamide (DMFA), was added to the RBCs. Results with appropriate solvent controls, 25 empty liposomes, and empty liposomes plus free-drug were also noted. Release of hemoglobin by hypotonic lysis of the same number of human RBCs by water was taken as a 100% positive control, while cells treated with PBS were taken as negative controls.

30 Preparations of L-RA comprising various lipids were incubated at a concentration of 20 microgram (ug) RA per ml with RBCs in PBS for 4 hr at 37°C. The toxicity of the L-RA preparations on the basis of percent RBC lysis is 35 shown in Table 2.

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TABLE 2

In Vitro Toxicity Of L-RA Preparations To RBCs

	LIPOSOME COMPOSITION	% RBC LYSIS
5	DMPC:Cholesterol 9:1	4.5
10	DMPC:Cholesterol 9:3	90.2
	DPPC	6.7
15	DMPC;SA:Cholesterol 8:1:1	70.4
	DMPC:DMPG 7:3	8
20	DMPC:DMPG 9:1	8.3

25 As may be seen from the data of Table 2, L-RA of DMPC:cholesterol, DPPC, DMPC:DMPG (7:3) and DMPC:DMPG (9:1) exhibited low RBC toxicity under these conditions. It is of interest to note that the latter two L-RA compositions exhibited superior encapsulation efficiencies

30 (Table 1).

A further experiment concerning the toxicity over time of free RA and L-RA (DMPC:DMPG-7:3) toward RBC was conducted. Human erythrocytes were incubated at 37°C in

35 PBS with 10 ug/ml free RA or 120 ug/ml L-RA, and RBC lysis monitored over a period of 5 hr. Figure 2 shows time courses of RBC lysis. At between about 1 hr and about 3 hr, the free RA extensively lysed a large majority of the erythrocytes. When a similar manipulation was performed

40 with L-RA (DMPC:DMPG(7:3) at a RA concentration of 120 ug/ml, little RBC lysis occurred (e.g., less than 10% after 6 hr).

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A study was also conducted concerning the effects upon RBC lysis in 2 hr of free RA and L-RA (DMPC:DMPG(7:3) at various concentrations. Figure 3 shows the results of this study. Free RA showed linearly increasing RBC lysis between about 5 ug RA/ml and about 30 ug RA/ml. Liposomal RA caused RBC lysis of only about 5% at a concentration of 160 ug RA/ml.

10

EXAMPLE 4

Acute Toxicity Of Free And Liposomal Retinoic Acid

15 The acute toxicity of free and liposomal all-trans retinoic acid was studied in CD1 mice. Free all-trans retinoic acid was prepared as an emulsion in normal saline containing 10% DMSO and 2% Tween 80 at a concentration of 3 to 5 mg/ml. Liposomal all-trans retinoic acid was
20 prepared using a lipid:drug ratio of 15:1. The final concentration of all-trans retinoic acid in the liposomal preparation was 3 mg/ml. Empty liposomes of the same lipid composition (DMPC;DMPG 7:3) were also tested at doses equivalent to 80 mg/kg, 100 mg/kg, and 120 mg/kg of
25 liposomal-all trans retinoic acid. Normal saline containing 10% DMSO and 2% Tween 80 was also tested as a control at a dose equivalent to 50 mg/kg of free all-trans retinoic acid. All drugs tested were injected intra-
venously via tail vein as a single bolus. The injected
30 volumes of free and liposomal-all-trans retinoic acid were the same for each dose.

Table 3 shows data obtained from these acute toxicity experiments.

35

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TABLE 3

Acute Toxicity of Free and
Liposomal All-Trans retinoic Acid

	Drug	Dose mg/kg	Number Animals with seizures	Number Animals alive (72 hr)
5				
10	Free RA	10	0/6	6/6
		20	6/6	5/6
		30	6/6	4/6
		40	3/3	0/3
15		50	3/3	0/3
	L-RA	40	0/6	6/6
		60	0/6	6/6
		80	0/6	6/6
20		100	0/6	6/6
		120	0/6	6/6
	Empty Liposomes	80	0/6	6/6
		100	0/6	5/6
25		120	0/6	6/6
	Normal saline			
	10% DMSO			
	2% Tween 80	50	0/6	6/6
30				

The maximum non-toxic dose of free all-trans retinoic acid was 10 mg/kg. Higher doses caused seizures immediately after injection. The acute LD₅₀ (deaths occurring up to 72 hours after injection) of free all-trans retinoic acid was 32 mg/kg. The cause of death was cardiopulmonary arrest after seizures for 1-2 minutes in all animals. No seizures or deaths were observed in the animals treated with liposomal all-trans retinoic acid at a dose of 120 mg/kg (maximum non-toxic dose and LD₅₀). Higher doses were not tested. No seizures were observed in the animals treated with empty liposomes or normal saline with 10% DMSO and 2% Tween 80.

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EXAMPLE 5

In Vitro Inhibition of Tumor Cell Growth

5 Liposomal all-trans retinoic acid (L-RA) was prepared
as described in Example 1.

Cells of the human monocytic cell line THP-1 were
inoculated into samples of eucaryotic cell culture medium
10 in the presence or absence of L-RA, at a final RA
concentration of 1 micromolar (μ M). After 24 hr at 37°C,
 3 H-thymidine was added to each culture and incorporation
thereof into cellular polynucleotides measured. Table 4
shows the percentage of tumor growth inhibition as
15 reflected by decreases in 3 H-thymidine incorporation
induced by L-RA of differing lipid compositions.

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TABLE 4

L-RA Inhibition of Tumor Cell Growth

5	LIPOSOME COMPOSITION	TUMOR CELL (THP-1) INHIBITION (%)
10	DMPC:Cholesterol 9:1	72
	DMPC:Cholesterol 9:3	22
15	DPPC	8
	DMPC;SA:Cholesterol 8:1:1	84
20	DMPC:DMPG 7:3	70
	DMPC:DMPG 9:1	32
25		

From Table 4, it should be noted that L-RA (DMPC:DMPG-7:3), which, as previously shown herein, gave a superior encapsulation efficiency and showed a low RBC toxicity (Tables 1 and 2), also effectively inhibited the tumor cell growth.

Cells of the human monocytic cell line THP-1 and of the human histiocytic cell line U-937 were inoculated at about 20,000 cells per cell in aliquots of eucaryotic cell culture medium contained in wells of a 96 well microtiter plate. The medium in various wells contained different amount of free RA or L-RA (DMPC:DMPG 7:3). The cells were incubated for 72 hr at 37°C and cell growth determined and compared to that of controls without any form of retinoic acid. Figure 4 shows the inhibition of THP-1 cell growth by increasing concentrations of free RA or L-RA (DMPC:DMPG

7:3). At concentrations of less than 1 ug RA/ml, both preparations inhibited cell growth by over 90%.

The human monocytic leukemia THP-1 cells, after a 72
5 hr incubation with either free RA or L-RA at a concentra-
tion of 0.3 ug RA/ml, were observed to have lost their
generally ovate form and to have a more flattened and
spread morphological appearance often associated with
cellular differentiation. The generally ovate form was
10 retained when the cells were cultured in the absence of
any free or liposomal retinoic acid.

After incubation for 24 hr with 0.3 ug/ml or 0.6
ug/ml RA or L-RA in another experiment, THP-1 cells had
15 increased levels of tissue transglutaminase enzymic
activity, or marker for monocytic cell differentiation.
As shown in Figure 5, THP-1 cells, at 4×10^6 cells/ml,
showed about 50% greater transglutaminase activity when
incubated with L-RA as compared to free RA at equivalent
20 retinoic acid concentrations.

Cells of the human histiocytic cell line U-937 were
distributed and cultured under the same conditions as the
THP-1 cells in the prior experiment. Figure 6 shows the
25 effects upon cell growth of increasing concentrations of
free all-trans retinoic acid (RA), liposomal (DMPC:DMPG
7:3) all-trans retinoic acid (L-RA) and empty liposomes
(which were devoid of retinoic acid). It should be noted
that the U-937 cells were almost completely growth-
30 inhibited by L-RA at a retinoic acid concentration of
about 10 ug/ml while this amount of free RA inhibited
growth less than 50%.

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EXAMPLE 6

Antitumor Activity of Liposomal All-Trans Retinoic Acid *in vivo*

5

The antitumor activity of liposomal-all trans retinoic acid (DMPC:DMPG 7:3) was tested in vivo against liver metastases of M5076 reticulosarcoma. C57BL/6 mice were inoculated with 20,000 M5076 cells on day 0. Intra-
10 venously treatment with 60 mg/kg liposomal all-trans retinoic acid was given on day 4. The mean survival of control animals (non-treated) was 21.8 + 1.6 days. The mean survival of treated animals was 27.0 ± 1.6 days.
15 Liposomal all-trans retinoic acid was shown, therefore, to have antitumor activity at a dose well below the maximum non-toxic dose, against a cell line (M5076) which was resistant to free retinoic acid in in vitro studies.
20 THP-1 cells treated in vitro with RA (1 MM) for 72 hours when injected subcutaneously into male mice, failed to develop into tumors, whereas untreated cells formed a huge mass of tumors in such mice.

EXAMPLE 7

25

Induction of Tissue Transglutaminase in Human Peripheral Blood Monocytes by Intracellular Delivery of Retinoids

30 Circulating blood monocytes are the precursors of macrophages which accumulate at the sites of tumor rejection [2], delayed hypersensitivity [25], chronic inflammation [6], and at the site of damaged tissue as a part of the healing processes [11] (see reference
35 citations in section D). At these sites, peripheral blood monocytes acquire new functional and biochemical characteristics that are associated with the maturation or

differentiation process. To understand clearly the mechanisms involved in differentiation, it is necessary to manipulate the extracellular environment and assess precisely a variety of cellular functions and biochemical activities.

Vitamin A and its analogues (retinoids) have been shown to exert a profound effect on the differentiation of monocytic cells. Both normal [19] and leukemic [7,17,28] 10 monocytic cells differentiate in response to retinoids which might suggest that retinoids play a role in regulating the differentiation of these cells. According to recent reports, the cellular activity of trans-glutaminase (TGase), an enzyme that catalyzes the covalent 15 cross-linking of proteins, may be directly linked to the retinoid's action [4,15,21,23,35,39,39]. Recently, the present inventors found that in vitro maturation of human peripheral blood monocytes (HPBM) to macrophage-like cells was associated with the induction and accumulation of a 20 specific intracellular TGase, tissue TGase [19,22]. Gamma (g)-interferon, which promotes the tumoricidal properties in HPBM, also augmented the expression of tissue TGase [19]. Similarly, the activation of guinea pig and mouse macrophages in vivo was associated with a marked increase 25 in tissue TGase activity [10,24,34]. Terminal differentiation of human monocytic leukemia cells (THP-1) induced by phorbol ester and retinoic acid was associated with induction and accumulation of tissue TGase (17), suggesting that the induction of tissue TGase was a marker 30 of monocytic cell differentiation. The present invention involves further definition of the role of retinoids in differentiation and maturation of HPBM and comprises studies of culture conditions that inhibit or facilitate the internalization of retinoids by HPBM on expression of 35 tissue TGase. The studies herein demonstrate that HPBM, isolated into two subpopulations, show no significant

difference in their ability to express tissue TGase activity induced by either in vitro culture or exposure to recombinant interferon gamma (rIFN- γ), and that the expression of tissue TGase in cultured HPBM may be induced
5 by a direct delivery of retinoids to intracellular sites.

A. Materials and Methods

1. Materials:

10 RPMI-1640 medium supplemented with L-glutamine and human AB serum were from Gibco Laboratories (Grand Island, NY); Escherichia coli-derived human recombinant γ -interferon (rIFN- γ) was kindly supplied by Genentech Inc. (South San Francisco, CA); and all-trans retinol (ROH) and all-trans retinoic acid (RA) were purchased from Sigma Chemical Co. (St. Louis, MO). The chromatographically pure lipids dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were from Avanti Polar Lipids, Birmingham, AL); tritiated putrescine (sp. act. 28.8 Ci/mmol), from New England Nuclear (Boston, MA); and tritiated ROH (sp. act. 15 mci/mmol), from Amersham (Arlington Heights, IL). Lipids, culture medium, and serum were screened for endotoxin with the Limulus
20 amebocyte lysate assay (MA Bioproducts, Walkersville, MD), and they were used only when endotoxin contamination was less than 0.25 ng/ml.

2. HPBM Isolation, Purification, and Culture

30 Pure populations of HPBM were obtained by counter-current centrifugal elutriation of mononuclear leukocyte-rich fractions obtained from normal donors who were undergoing routine plateletapheresis [12]. HPBM were
35 isolated into two subpopulations according to size with a Coulter ZBI counter and C-1000 channelizer (Coulter

Electronics, Hialeah, FL). The median volume of small monocytes was 255 mm^3 , and that of the large monocytes was 280 mm^3 . The small monocytes were $95\% \pm 3\%$ nonspecific esterase-positive and the large monocytes were $98\% \pm 2\%$ positive. Detailed procedures for isolation and characteristics of these subpopulations have been published elsewhere [36,37]. Small, large, or mixed (obtained by mixing equal parts of small and large HPBM) HPBM subpopulations were washed once with medium (RPMI-1640 supplemented with L-glutamine, 20 mM HEPES buffer, 20 ug/ml gentamicin, and 5% human AB Serum) and resuspended to 0.5 million/ml density in the same medium. The cells were dispensed in 4-ml samples into 35-mm-well plates and cultured under appropriate conditions.

15

3. Enzyme Assay

Tissue TGase activity in cell extracts was measured as a Ca^{2+} , dependent incorporation of [^3H] putrescine into dimethylcasein. In brief, cultured HPBM were washed three times in Tris-buffered saline (20 mM Tris-HCl, 0.15 M NaCl, pH 7.6) and scraped from the dish in a minimal volume of the same buffer containing 1 mM EDTA and 15 mM Beta-mercaptoethanol. The cells were lysed by sonication, and TGase activity in the lysates was determined as described previously [13,20]. The protein content in cell lysates was determined by Lowry's method [14] with bovine gamma globulin as standard. The enzyme activity was expressed as nanomoles of putrescine incorporated into dimethyl-casein per hour per milligram of cell protein.

4. Immunochemical Detection of Tissue TGase

To detect tissue TGase in cell extracts, the cell lysates were solubilized in 20 mM Tris-HCl (pH 6.8) containing 1% sodium dodecyl sulfate (SDS), 0.75 M Beta-

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mercaptoethanol, 2.5% sucrose and 0.001% bromophenol blue. Solubilized extracts were fractionated by electrophoresis on a 6.5% discontinuous polyacrylamide gel and electroblotted onto nitrocellulose paper. The paper was
5 neutralized with 5% bovine serum albumin and treated with iodinated anti-tissue TGase antibody; the preparation, characterization and properties of this antibody have been described elsewhere [24]. The unbound antibody was removed by washing the paper in Tris-HCl buffer (50 mM, pH
10 7.5) containing 200 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 0.25% gelatin, and the paper was dried and autoradiographed as described earlier [20,24].

5. Preparation of Liposomes

15 Multilamellar vesicles (liposomes) containing DMPC and DMPG at a molar ratio of 7:3 were prepared as described [16,18]. All-trans ROH or RA were encapsulated by adding the required amount of the drug (predissolved in
20 ethanol) in lipid-containing organic solvents before vacuum drying. The dried lipid-drug film was dispersed by agitation in sterile saline solution. Retinoids up to a 1:10 drug:lipid ratio could be completely encapsulated within the liposomes and were highly stable. The
25 stability and encapsulation efficiency of the liposome preparations were studied by using radiolabelled retinol and showed that only 5% \pm 2% of the incorporated radioactivity leaked out in the supernatant after 24-hr incubation at 37°C.

30

6. Binding Assay for [³H]ROH

Freshly isolated HPBM were cultured in serum containing medium alone or medium plus 50 units (U)/ml
35 rIFN- γ for varying periods of time. At the end of indicated time periods, HPBM monolayers were washed twice.

in ice cold medium and resuspended in 0.5 ml of prechilled reaction mixture containing 5.0 microcuries (μCi)/ml [$11,12(\text{n})^3\text{H}$] vitamin A (free ROH) in RPMI medium supplemented with 5% delipidized human AB serum (serum 5 delipidization was done by organic solvent extraction as described earlier [33]. Binding assays were carried out for 1 hr in an ice bath. After a 1-hr incubation, the monocyte monolayers were washed six times with ice-cold medium and the cells were lysed in 200 μl of Triton X-100. 10 Fifty-microliter aliquots of cell lysates, in triplicate, were counted for the cell-associated radioactivity. Background counts, obtained by adding the reaction mixture toward the end of the 1-hr incubation before harvesting, were subtracted from the experimental values.

15

B. Results

1. Tissue TGase Induction During In Vitro Culture of HPBM

20

The culture of HPBM in the presence of serum-containing medium for up to 10 days was associated with a marked induction of tissue TGase activity in both small and large HPBM (Fig. 7), the increase in enzyme activity 25 being more rapid after about 4 days of culture. After 10 days in culture, small monocytes showed a 93-fold increase in enzyme activity (from 0.44 to 41.1 nmol/hr/mg), whereas large HPBM accumulated about 103-fold increase in the enzyme activity (from 0.36 to 37.4 nmol/hr/mg). Small and 30 large HPBM mixed together and cultured under similar conditions showed no significant difference in the rate and amount of accumulation of tissue TGase activity compared with that of individual HPBM fractions (data not shown). Induction of enzyme activity was associated with 35 a change in the morphology of cultured monocytes. Freshly isolated HPBM looked rounded, but after 6-8 days in

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culture both the large and small HPBM became firmly adherent to the plastic surface, were more spread and flattened, and had the appearance typical of mature macrophages. By day 10, when the cells had accumulated maximal levels of enzyme activity, these levels then either plateaued or started declining.

2. Effect of rIFN- γ on Tissue TGase Expression

The effect of continuous exposure to rIFN- γ on induction of tissue TGase activity in HPBM is shown in Figure 8. Small and large monocytes were cultured in serum-containing medium for 72 hr in the presence of increasing concentrations of rIFN- γ . Enzyme activity in the HPBM populations increased significantly after their continuous exposure to rIFN- γ compared with that of cells cultured in the presence of medium alone. However, rIFN- γ dose size produced no significant difference in enzyme activity between the two HPBM populations. As previously noted [19], a 100-U/ml dose of rIFN- γ seemed to be optimal for augmenting TGase activity; higher rIFN- γ -concentrations were less effective. The inductive effect of rIFN- γ on tissue TGase activity was evident at 5 U/ml and pretreatment of HPBM cultures with rIFN- γ (100 U/ml) followed by washing and subsequent culture in medium alone did not enhance the expression of tissue TGase. The rIFN- γ -induced augmentation of tissue TGase was associated with morphologic changes in HPBM so that the rIFN- γ -treated cells were more spreadout and flattened than the untreated control cells after three days in culture.

3. Effect of Retinoids on Tissue TGase Induction

Since the two HPBM populations showed no heterogeneity in terms of induced tissue TGase levels, our subsequent studies were done with whole HPBM fraction

without separation into subsets. HPBM cultured in the presence of 500 nM RA for 24 hr accumulated at least three-fold higher enzyme activity than did the control cells cultured in medium along (Fig. 9). Continuous exposure to RA caused a rapid and linear increase in the enzyme activity, whereas in the control cells no significant change in the level of tissue TGase activity was observed for up to 2 days of culture. By day 3, the control cells accumulated about six-fold higher enzyme activity (3.4 nmol/hr/mg) than did freshly isolated HPBM (0.6 nmol/hr/mg), but they still had significantly less enzyme activity than the RA-treated cells (9.8 nmol/hr/mg). Retinoic acid-induced expression of tissue TGase was dose dependent (Fig 9 inset). ROH, the physiologic analogue of RA, did not induce the expression of tissue TGase in HPBM even at a dose of 1 uM. Thus, HPBM cultured in the presence of ROH for up to 3 days showed no significant difference in accumulation of tissue TGase activity when compared with that of control cells cultured in medium along (Fig. 9).

4. Effect of Liposome-Encapsulated Retinoids on Tissue TGase Induction

Liposome-encapsulated RA was more effective in inducing tissue TGase expression than was free RA at an equimolar concentration. After 24-hr culture, the amount of tissue TGase activity in HPBM induced by free or liposomal RA at an equimolar concentration of 500 nM was not significantly different (3.4 and 3.7 nmol/hr/mg, respectively); after 48 and 72 hr, however, liposomal RA-treated cells accumulated at least 50% more enzyme activity than did free RA-treated cells (Fig. 10A). That increase in enzyme activity by liposome-encapsulated RA was a specific effect of RA and not of lipids was demonstrated by the fact that a culture of HPBM in the

presence of "empty liposomes," and containing equivalent amount of lipids did not induce enzyme activity throughout the incubation period. "Empty liposomes," as reported earlier [20], inhibited serum-induced expression of tissue TGase after 72 hr of culture (Fig 10A). The free or liposomal RA-induced increase in enzyme activity was caused by an increased amount of the enzyme peptide, as revealed by Western-blot analysis of cell lysates using a iodinated antibody to tissue TGase (Fig. 10B). The increase in enzyme activity was proportional to the increase in enzyme peptide and not caused by activation of preexisting enzyme.

Retinol, which in its free form was unable to enhance the expression of tissue TGase in HPBM, became active when presented in liposomal form. Liposome-encapsulated ROH caused a rapid and linear increase in tissue TGase activity with time in culture (Fig 11A). After 72 hr of culture, liposomal-ROH caused a nine-fold increase in enzyme activity (7.1 nmol/hr/mg) when compared to that of control cells exposed to free ROH under similar conditions (0.8 nmol/hr/mg). Liposomal ROH-induced expression of tissue TGase resulted from increased accumulation of the enzyme peptide as demonstrated by Western-blot analysis (Fig. 11B).

5. Tissue TGase induction is Related to HPBM Uptake of Retinoids

The effect of in vitro maturation and rIFN- γ treatment on the binding of tritiated-ROH by HPBM was examined. After 4 days of control culture (medium dose), tritiated-ROH binding by HPBM increased 50% compared to this binding by freshly isolated cells. After 9 days the control culture binding value increased to 350%. The increases in

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ROH binding were associated with parallel increases in tissue TGase activity (Table 5).

TABLE 5

		Days in culture	$[^3\text{H}]$ ROH bound ^b (cpm/10 μg protein)	Tissue TGase activity (nmol/hr/mg)
Culture conditions ^a				
Medium alone	0	684 \pm 25	0.25 \pm 0.13	
	4	994 \pm 115	2.96 \pm 0.75	
	9	2,220 \pm 144	32.60 \pm 8.50	
Medium alone	3	626 \pm 37	2.9 \pm 0.23	
Medium + rIFN- γ	3	1,782 \pm 130	7.6 \pm 0.7	

^aHPBM were cultured in serum-containing medium alone or medium containing 50 U/ml rIFN- γ for indicated periods of time.

^bBinding of tritiated ROH during different periods of culture was determined as described in Materials and Methods.

^cParallel cultures of HPBM maintained under similar conditions were used for assaying enzyme activity as described in Materials and Methods.

Exposure of HPBM to rIFN- γ augmented the ROH binding and the expression of enzyme activity. The rIFN- γ -treated cells showed a threefold higher $[^3\text{H}]$ ROH binding than did control cells incubated in the presence of serum-containing medium alone for the same period of time. The presence of delipidized serum in the reaction mixture was essential; only 10% of the total counts were cell-associated when delipidized serum was omitted from the reaction mixture.

C. Discussion

The results reported in this Example suggested that HPBM, isolated into two populations based on their size and density, have equal potential to differentiate into mature macrophages. The in vitro maturation of HPBM to macrophages was associated with enhanced binding and uptake of retinol, presumably as a result of the acquisition of cell surface receptors for serum retinol-binding protein. Exposure of HPBM to rIFN- γ for 72 hr led to enhanced binding of [3 H]ROH that was comparable to the binding activity of control HPBM cultured in vitro for 9 days. HPBM maturation induced by in vitro culture or by exposure to rIFN- γ was accompanied by similar morphologic and enzymatic changes. The requirement of cell surface receptor for serum retinol-binding protein could be circumvented by direct intracellular delivery of ROH.

Recently, several reports have suggested an association between monocytic cell differentiation and induction of tissue TGase [10,17,19,21-24,34]. Freshly isolated HPBM that have very low levels of tissue TGase accumulate large amounts of this enzyme after their in vitro maturation [19,22]. Just as the two subpopulations of HPBM showed no significant difference in their ability to induce and accumulate tissue TGase activity during in vitro differentiation to macrophages, both fractions were equally responsive to the effect of rIFN- γ in terms of augmented enzyme expression (Fig 8). Functional heterogeneity among HPBM subpopulations isolated by similar criteria has been reported earlier. Thus, the subsets of HPBM isolated into small and large populations have been reported to produce different amounts of reactive oxygen species [37], prostaglandins [1,30], antibody dependent cell-mediated cytotoxicity [27], and tumor-cell killing [26]. This functional

heterogeneity among HPBM subpopulations has been attributed to either maturational or clonal differences. The data presented herein, however, suggest no heterogeneity among HPBM subpopulations in induction of tissue TGase, a marker for monocytic cell differentiation, and equal potential for differentiating into mature macrophages. The ability of rIFN- γ to enhance tissue TGase expression in both HPBM subpopulations suggests that this endogenous cytokine may play an important role in the maturation, differentiation, and expression of differentiated functions in monocytic cells.

The factors in serum responsible for induction and accumulation of tissue TGase in cultured HPBM and macrophages have been shown to be endogenous retinoids and serum retinol-binding protein [21]. Extraction of retinoids by delipidization or depletion of retinol-binding protein from the serum completely abolished its enzyme-inducing ability [19,21]. Serum retinol-binding protein is believed to be responsible for intravascular transport and delivery of retinol to specific target tissues [8,9,29,31]. Receptors for serum retinol-binding protein present on the surface of target cells are responsible for the specificity of the delivery process [9,31]. The binding of ROH-retinol-binding protein complex to cell surface receptors apparently facilitates the delivery of ROH into the interior of the cell [9,31]. At supraphysiologic doses (greater than 10 nM) on the other hand, RA can enter the cells directly by simple diffusion without the participation of surface receptors for retinol-binding protein [21]. This suggested that freshly isolated HPBM probably lack the cell surface receptors for serum retinol-binding protein and therefore cannot internalize the endogenous or exogenous retinoids. Indeed, the addition of exogenous RA to HPBM cultures at doses (e.g. greater than 10 nM) at which the receptor-

mediated delivery becomes irrelevant resulted in a marked induction of tissue TGase activity (Fig. 9). The enzyme-inducing ability of RA was augmented further by encapsulating RA within the liposomes and allowing its 5 internalization via phagocytosis (Fig. 10).

Of particular interest was the effect of ROH, which, in its free form did not induce the expression of tissue TGase in freshly isolated HPBM. When ROH was encapsulated 10 within liposomes, however, the requirement for a cell surface receptor for serum retinol-binding protein was obviated. Thus liposomal ROH induced a significant level of tissue TGase activity in HPBM (Fig. 11). This suggested an effective approach for targeting retinol or 15 its inactive analogues to the monocytic cells with no or minimal toxic effects. Because HPBM lack cell surface receptors for serum retinol-binding protein makes administered ROH subject to nonspecific internalization by other cell types. The present studies suggested, further- 20 more, that interaction of ROH-retinol binding-protein complex with the cell surface receptor is required only for the intracellular delivery of retinol and that, unlike in the case of other hormones [3], ligand-receptor inter- action may not require a second messenger for expression 25 of the final event. The increase in TGase enzyme activity induced by free RA or liposome-encapsulated RA or ROH, was the result of the accumulation of enzyme protein rather than the activation of preexisting enzyme, as revealed by immunoblots of the cell lysates using an iodinated anti- 30 body to tissue TGase (Figs. 10,11).

Preliminary data on tritiated ROH-binding (Table 5) further supported the concept that in vitro differentia- 35 tion of HPBM to mature macrophages was associated with acquisition of cell surface receptors for retinol-binding protein and that treatment with rIFN- γ augmented the

expression of these receptors. Once the HPBM acquire these receptors, they could internalize the endogenous retinoids and induce the expression of tissue TGase. Indeed, retinoids have been shown specifically to trigger 5 the gene for tissue TGase in myelocytic cells [23].

Impairment of macrophage function in retinoid-deficient animals has been well documented to lead to increased incidence of infections and decreased tumor-cell 10 killing [5]. In cultures of guinea pig peritoneal macrophages, RA has been reported to increase the intracellular levels for the tumoricidal enzyme arginase [32]. The present findings that retinoids play an important role 15 in the differentiation process of HPBM support the idea that retinoids are the important regulators of monocyte/macrophage functions.

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* * * * *

Changes may be made in the construction, operation
5 and arrangement of the various steps and procedures and
components such as various retinoids or lipids described
herein without departing from the concept and scope of the
invention as defined in the following claims.

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CLAIMS:

1. A method for therapeutic administration of retinoids
5 to an animal, the method comprising:

preparing liposomes comprising phospholipid and
retinoid; and

10 administering a quantity of said liposomes to the
animal, said quantity containing a
therapeutically effective amount of the
retinoid.

15 2. The method of claim 1 wherein the liposomes are
administered parenterally.

20 3. The method of claim 1 wherein the liposomes are
administered topically.

25 4. The method of claim 1 wherein the liposomes are
administered orally.

5. The method of claim 1 wherein the liposomes are
administered intraperitoneally.

30

6. The method of claim 1 wherein the retinoid is
retinoic acid.

35

7. The method of claim 1 wherein the retinoid is all trans-retinoic acid.

5 8. The method of claim 1 wherein the retinoid is
retinol.

9. The method of claim 1 wherein the retinoid is all
10 trans-retinol.

10. The method of claim 1 wherein the retinoid is selected from the group consisting of:

15 all-trans-retinoic acid, retinoic acid methyl ester,
retinoic acid ethyl ester, phenyl analog of
retinoic acid, etretinate, retinol, retinyl
acetate, retinaldehyde, and 13-cis-retinoic
20 acid.

11. The method of claim 1 wherein the phospholipid is selected from a group consisting of:

25 phosphatidylcholine, phosphatidic acid, phosphatidylserine, phosphatidylglycerol, sphingolipids,
sphingomyelin, cardiolipin, glycolipids,
cerebrosides, phosphatides, dimyristoylphosphatidyl-
30 choline, dimyristoylphosphatidylglycerol, egg
phosphatidylcholine, diacyloylphosphatidylcholine,
dipalmitoylphosphatidylcholine,
distearoylphosphatidylcholine, 1-myristoyl-2-
palmitoylphosphatidylcholine, 1-palmitoyl-2-
35 myristoyl phosphatidylcholine, 1-palmitoyl-2-
stearoyl phosphatidylcholine, 1-stearoyl-2-palmitoyl

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phosphatidylcholine, dioleoylphosphatidylcholine,
dilauryloylphosphatidylglycerol, dipalmitoyl-
phosphatidylglycerol, distearoylphospha-
tidylglycerol, dioleoylphosphatidylglycerol,
5 dimyristoylphosphatidic acid, dipalmitoyl
phosphatidic acid, dimyristoyl phosphatidyl-
ethanolamine, dipalmitoyl phosphatidylethanolamine,
dimyristoyl phosphatidylserine, dipalmitoyl
phosphatidylserine, brain phosphatidylserine, brain
10 sphingomyelin, dipalmitoyl sphingomyelin, and
distearoyl sphingomyelin.

12. The method of claim 1 wherein the phospholipid
15 comprises dimyristoylphosphatidylcholine and dimyristoyl-
phosphatidylglycerol.

13. The method of claim 12 wherein the dimyristoyl-
20 phosphatidylcholine and dimyristoylphosphatidylglycerol
are in about a 7:3 ratio.

14. The method of claim 1 wherein the animal being
25 administered the liposomes bears a tumor impeded by
retinoids and the administering step serves to impede
growth of said tumor.

30 15. The method of claim 1 wherein the animal being
administered the liposomes has a dermatological disorder
responsive to retinoids and the administering step results
in clinical improvement of said disorder.

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16. The method of claim 1 wherein the animal being administered the liposomes has a ophthalmic disease responsive to retinoids and the administering step results in clinical improvement of said disease.

5

17. The method of claim 1 wherein the animal being administered the liposomes has a rheumatic disease responsive to retinoids and the administering step results in clinical improvement of said disease.

18. The method of claim 1 wherein the animal being administered the liposomes has a vitamin A deficiency responsive to retinoids and the administering step results in clinical improvement of said deficiency.

19. The method of claim 14 wherein the retinoid is all-trans retinoic acid.

20. A method for therapy of an animal afflicted with cancer, the method comprising:

25

identifying an animal afflicted with cancer;

preparing liposomes comprising phospholipid and a retinoid; and

30

parenterally administering a quantity of said liposomes to the animal, said quantity containing a therapeutically effective amount of the retinoid.

35

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21. A method for cancer chemotherapy of an animal, the method comprising:

identifying an animal afflicted with cancer;

5

preparing liposomes comprising phospholipid and retinoic acid; and

10 parenterally administering a quantity of said liposomes to the animal, said quantity containing a therapeutically effective amount of the retinoic acid.

15 22. A method for inducing the in vivo differentiation of peripheral blood monocytes and other cells, the method comprising the steps of:

20 preparing liposomes comprising phospholipid and retinoid; and

25 parenterally administering a quantity of said liposomes to an animal, said quantity containing an amount of the retinoid effectively inducing peripheral blood monocyte differentiation.

30 23. The method of claim 20, 21, or 22 wherein the retinoid is retinoic acid.

35 24. The method of claim 23 wherein the retinoic acid is all-trans retinoic acid.

35

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25. The method of claim 20, 21, or 22 wherein the retinoid is retinol.

5 26. The method of claim 25 wherein the retinoid is all-trans retinol.

10 27. The method of claim 20, 21, or 22 wherein the phospholipid comprises dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol.

15 28. The method of claim 27 wherein the dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol are in about a 7:3 ratio.

29. A process for producing a powder which forms
20 liposomes comprising a retinoid upon suspension in an aqueous solution, said process comprising the steps of:

- (a) dissolving retinoid in t-butanol to form a first solution;
- 25 (b) mixing the first solution and a phospholipid to dissolve the phospholipid and form a second solution; and
- 30 (c) lyophilizing the second solution to produce a powder.

30. The process of claim 29 wherein the phospholipid is a
35 phospholipid film.

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31. A composition of matter produced by a process comprising the steps of:

- 5 (a) dissolving retinoid in t-butanol to form a first solution;
- 10 (b) mixing the first solution and a phospholipid to dissolve the phospholipid and form a second solution; and
- 15 (c) lyophilizing the second solution to produce a powder.

15 32. The composition of claim 31 wherein the phospholipid is a phospholipid film.

20 33. The composition of claim 31 wherein the retinoid is all-trans retinoic acid, all-trans retinol or a derivative thereof.

25 34. The composition of claim 31 wherein the retinoid is all-trans retinoic acid.

30 35. The composition of claim 31 wherein the retinoid is all-trans retinol.

35 36. The composition of claim 31 wherein the phospholipid is one or more of phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, sphingomyelin, and phosphatidic acid.

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37. The composition of claim 31 wherein the phospholipid comprises DMPC and DMPG.

5 38. The composition of claim 31 wherein the phospholipid consists essentially of DMPC and DMPG in 7:3 ratio.

10 39. The composition of claim 31 wherein the powder is defined further as having a ratio of retinoid to phospholipid between about 1:5 and about 1:50.

15 40. The composition of claim 31 wherein the powder is defined further as having a ratio of retinoid to phospholipid between about 1:10 and about 1:15.

20 41. A process for producing an aqueous suspension of liposomes comprising a retinoid, said process comprising the steps of:

- (a) dissolving retinoid in t-butanol to form a first solution;
- 25 (b) mixing the first solution and a phospholipid to dissolve the phospholipid and form a second solution;
- 30 (c) lyophilizing the second solution to produce a powder; and
- (d) agitating a sample of the powder in an aqueous solution.

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42. The process of claim 41 wherein the phospholipid is a phospholipid film.

5 43. A process for treating an animal with retinoids, said process comprising the steps of:

(a) dissolving retinoid in t-butanol to form a first solution;

10

(b) mixing the first solution and a phospholipid to dissolve the phospholipid film and form a second solution;

15

(c) lyophilizing the second solution to produce a powder;

(d) agitating a sample of the powder in an aqueous solution to produce a liposome suspension; and

20

(e) administering the liposome suspension to the animal.

25 44. The process of claim 43 wherein the phospholipid is a phospholipid film.

45. The method of claim 44 wherein the suspension is
30 administered parenterally.

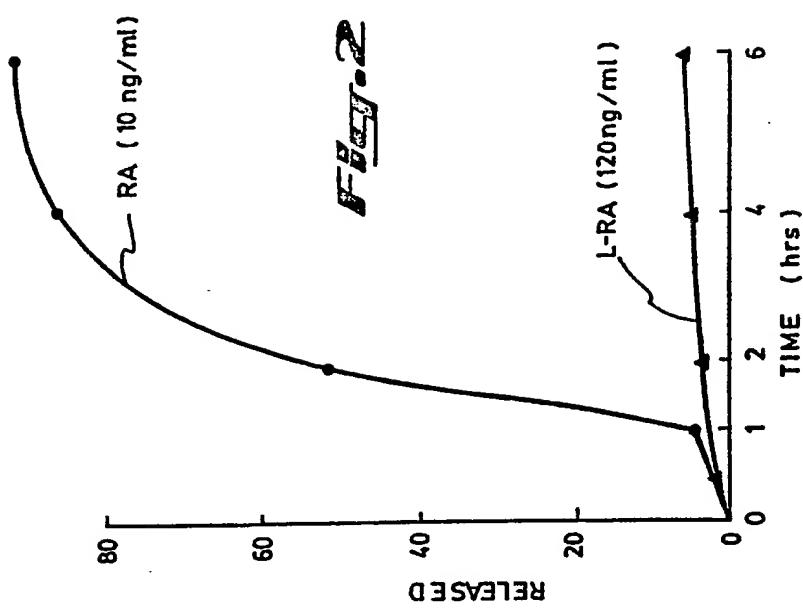
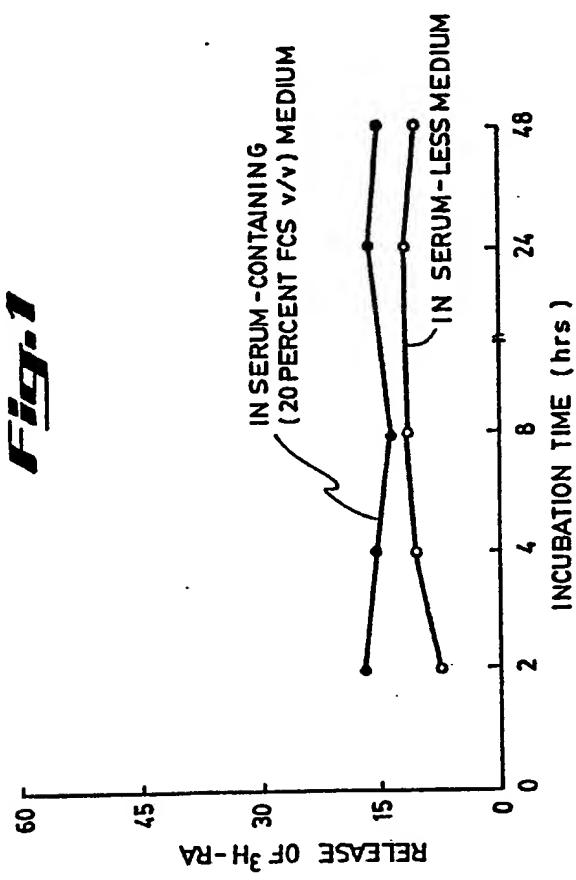
46. The method of claim 44 wherein the suspension is
administered topically.

-53-

47. The method of claim 44 wherein the suspension is administered orally.

5 48. The method of claim 44 wherein the suspension is administered intraperitoneally.

STABILITY OF ^{3}H -RETINOIC ACID IN
PRESENCE OR ABSENCE OF SERUM
PROTEINS AFTER ENCAPSULATION
IN LIPOSOMES.



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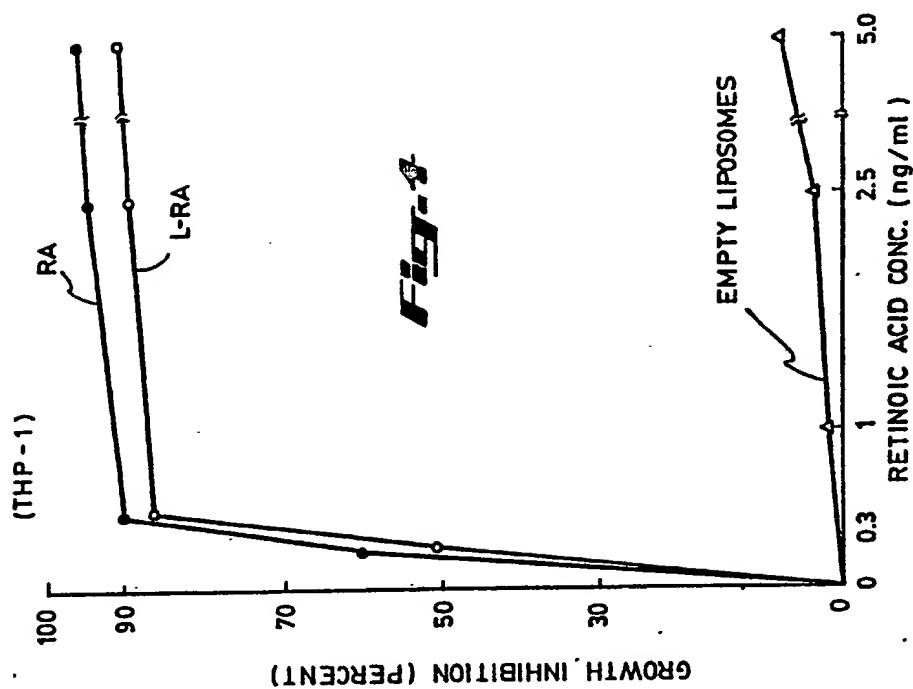
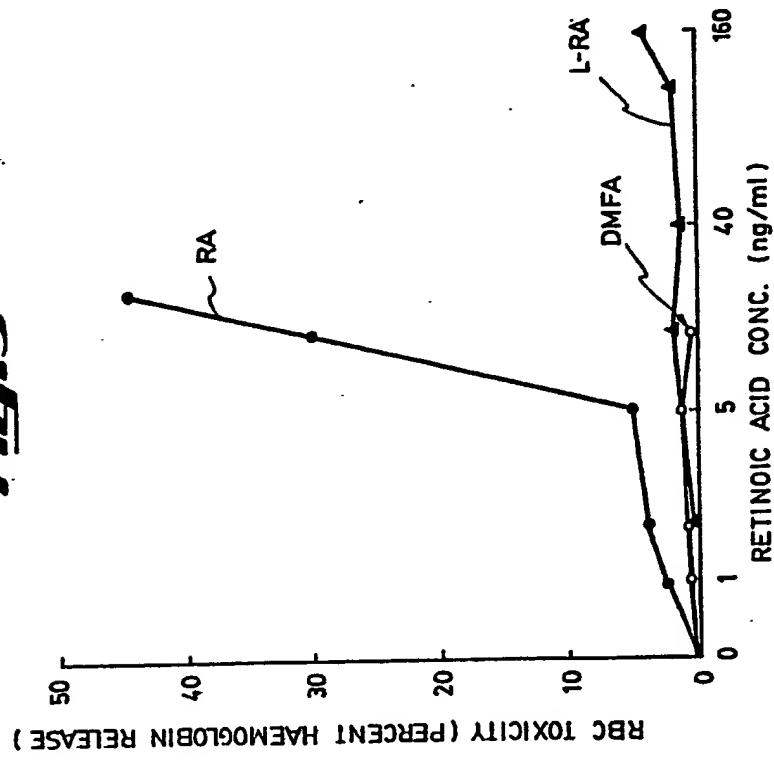
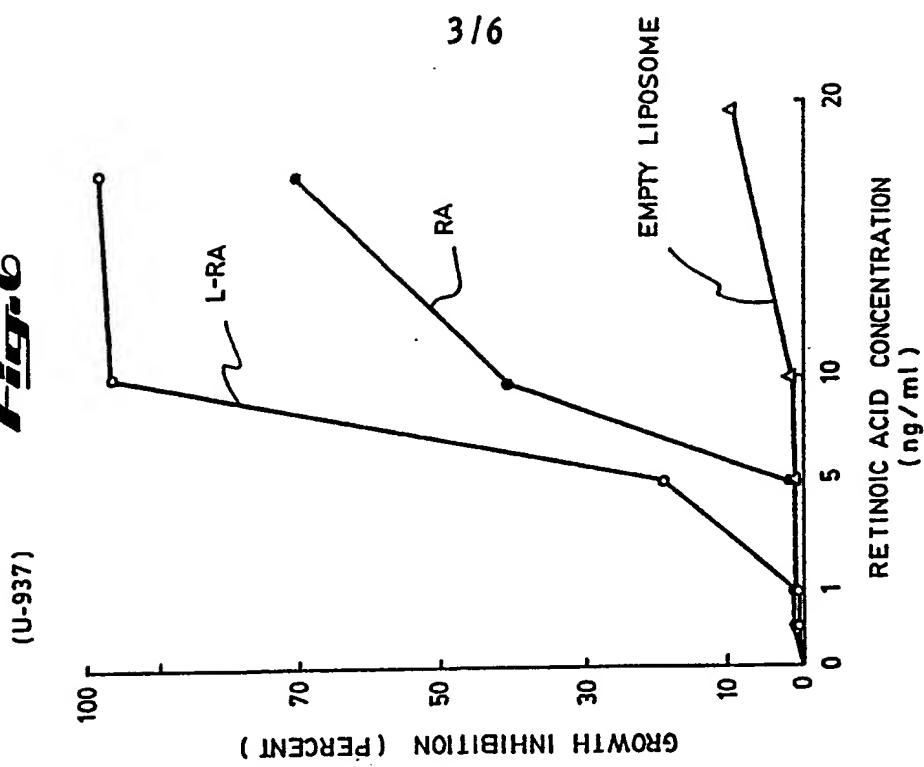
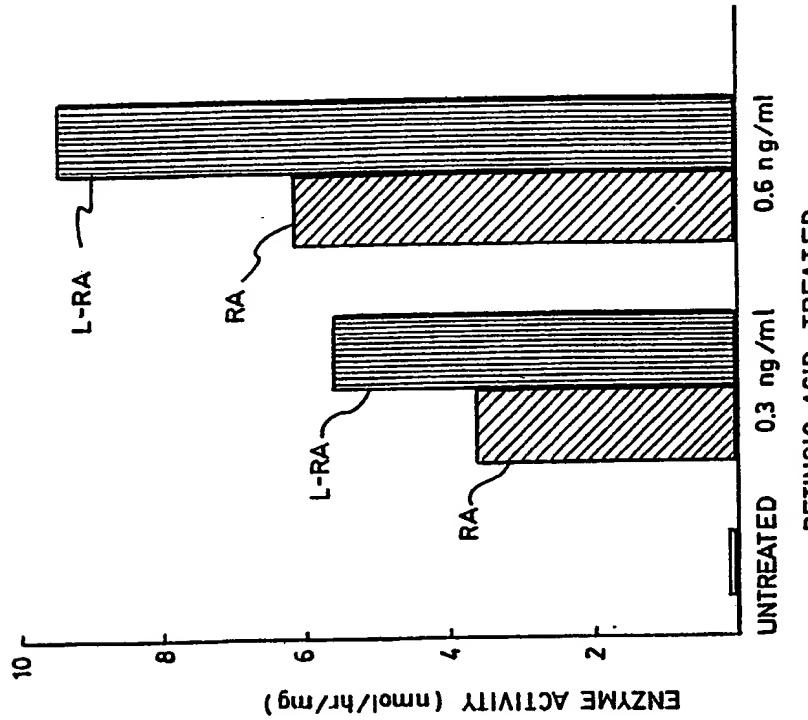
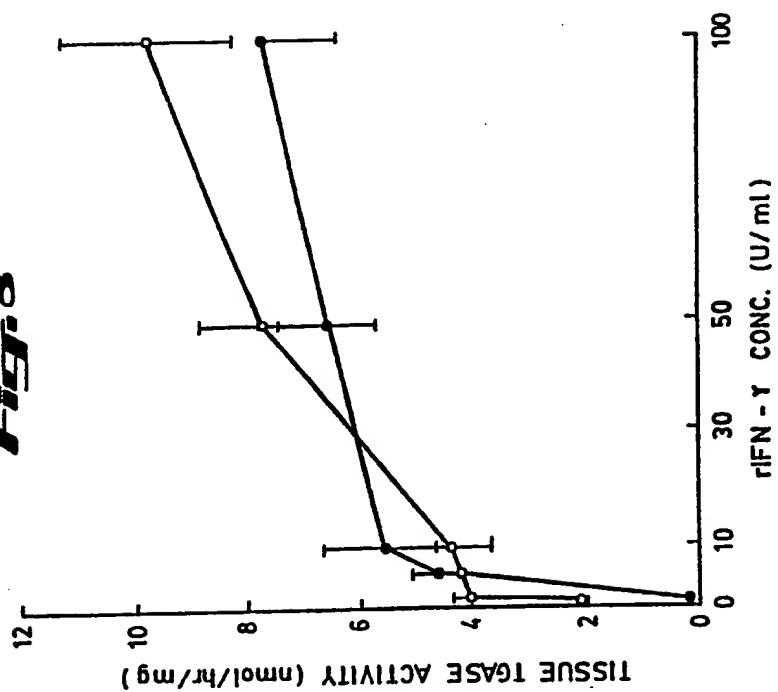
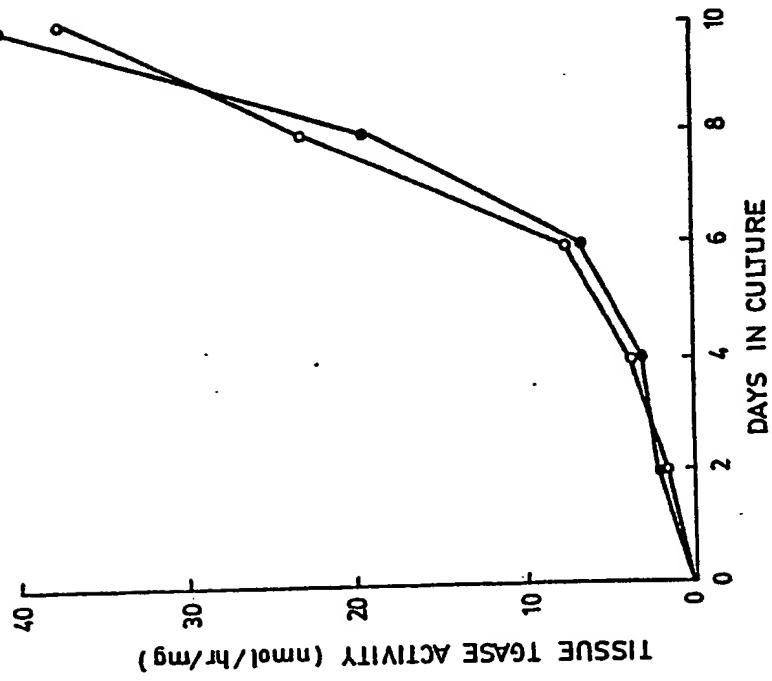
Fig. 2*Fig. 3*

Fig. 6*Fig. 5*

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Fig. 8*Fig. 7*

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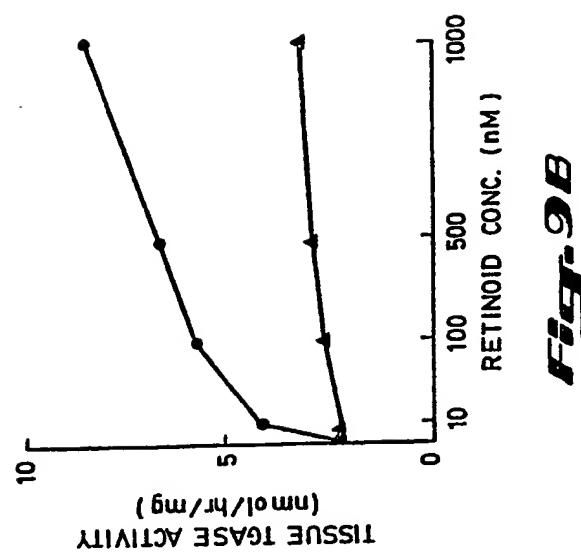


Fig. 9B

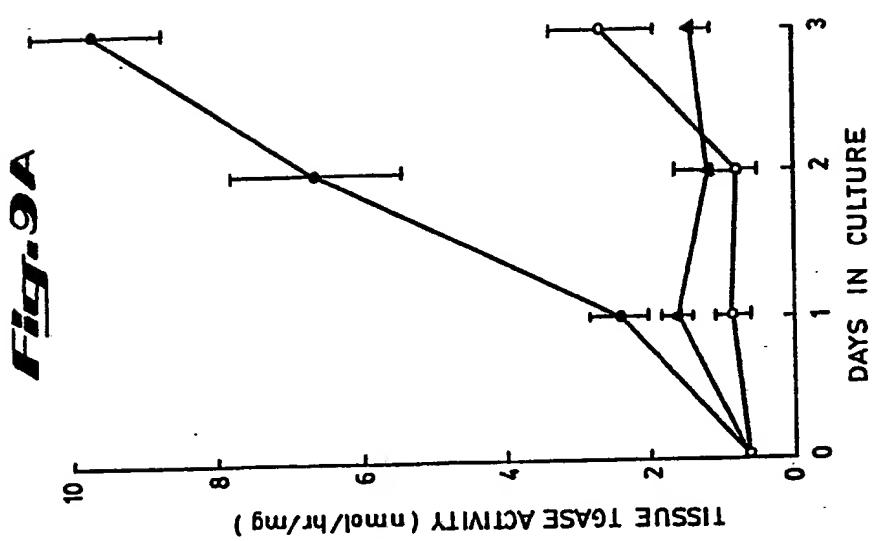
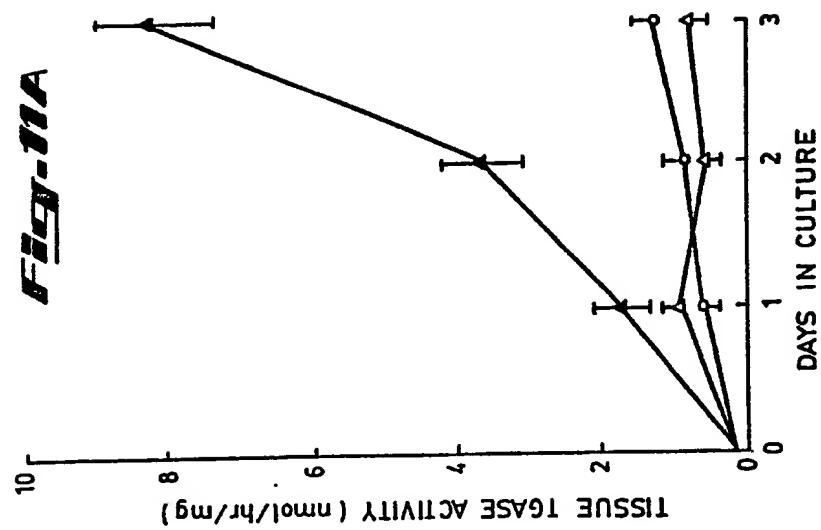
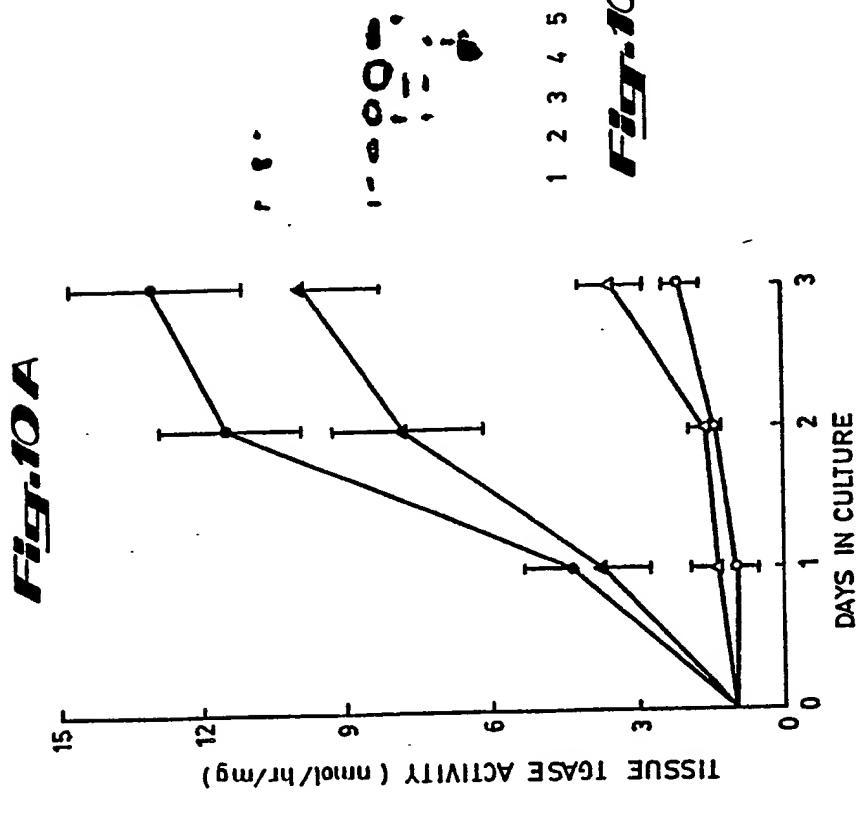


Fig. 9A

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Fig. 11B*Fig. 10B*

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 89/00435

I. CLASSIFICATION & SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC4: A 61 K 47/00, 31/595

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC4	A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	GB, A, 1575343 (IMPERIAL CHEMICAL INDUSTRIES LIMITED) 17 September 1980, see especially page 1 col 1 line 27 and page 2 col 1 line 8-61. ---	29-37, 39-42
Y	Thermochimica Acta, Vol. 122, 1987 Salvatore Gurrieri and Francesco Castelli: "Thermotropic behaviour of dipalmitoylphosphatidylcholine liposomes containing retinoids ", see page 117 - page 122 see especially page 118 "Preparation of liposomes" ---	29-37, 39-42
Y	Biochimica et Biophysica Acta, Vol. 688, 1982 William Stillwell et al: "Effect of retinol and retinoic acid on permeability, electrical resistance and phase transition of lipid bilayers ", see page 653 - page 659 see especially table 1 ---	29-37, 39-42

* Special categories of cited documents: ¹⁴

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search
8th May 1989

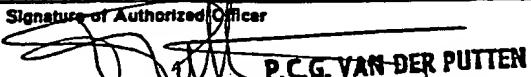
Date of Mailing of this International Search Report

19.05.89

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer



P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A1, 0274174 (YISSUM RESEARCH AND DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM) 13 July 1988, see especially page 12 --	29-42
A	EP, A2, 0190926 (STERWIN AG.) 13 August 1986, see especially column 4 line 47-54 and page 5 line 52-67 --	29-42
A	EP, A3, 0170642 (AKTIEBOLAGET DRACO) 5 February 1986, see especially page 7-8 --	29-42
A	EP, A1, 0229561 (MOET-HENNESSY RECHERCHE) 22 July 1987, see especially claim 1 --	29-42
A	Biochimica et Biophysica Acta, Vol. 944, 1988 Göran Fex and Gunvor Johansson: "Retinol transfer across and between phospholipid bilayer membranes ", see page 249 - page 255 see especially page 250 column I --	29-42
A	WO, A1, 88/03018 (LIPOSOME TECHNOLOGY, INC.) 5 May 1988, see the whole document -----	29-42

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹**

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers 1-28, because they relate to subject matter not required to be searched by this Authority, namely:
43-48

Methods for treatment of the human body by therapy [PCT Rule 39 (iv)]

2. Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this International application as follows:

1. As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2. As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
 No protest accompanied the payment of additional search fees.